**Ca**²⁺-permeable and Ca²⁺-impermeable AMPA receptors coexist on horizontal cells

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Fura-2 fluorescent calcium imaging was used for analyzing the subtype of AMPA receptors in freshly dissociated horizontal cells of carp retina. Exogenous application of AMPA induced an increase of intracellular concentration of free Ca²⁺ ([Ca²⁺]) in horizontal cells, while the [Ca²⁺] increase was partly inhibited by nifedipine. The residual [Ca²⁺] increase was completely eliminated by joro spider toxin-3, a blocker of Ca²⁺-permeable AMPA receptors. On the other hand, the application of pentobarbital, which blocked Ca²⁺-impermeable AMPA receptors, could also partly inhibit the increase of [Ca²⁺], implying that the application of AMPA induced the activation of both Ca²⁺-permeable and Ca²⁺-impermeable AMPA receptors and the consequent activation of voltage-gated Ca²⁺ channels. Taken together, these results suggested that Ca²⁺-permeable and Ca²⁺-impermeable AMPA receptors were coexpressed on horizontal cells. NeuroReport 16:263–266 © 2005 Lippincott Williams & Wilkins.

**Key words:** AMPA receptor; Calcium imaging; Horizontal cell; Joro spider toxin; Pentobarbital

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**INTRODUCTION**

The majority of fast excitatory synaptic transmission in the central nervous system is mediated by AMPA-type glutamate receptors (AMPArs) [1]. AMPARs are assembled from four subunits, GluR1 through GluR4, either alone or in various combinations. The functional properties of AMPARs depend on their subunit composition. AMPARs possessing the GluR2 subunit exhibit little Ca²⁺ permeability, whereas receptors lacking GluR2 show high Ca²⁺ permeability [2,3]. The control of Ca²⁺ permeability in AMPA receptor subunits was traced to a single amino acid in the second membrane-associated domain (transmembrane segment M2), the so-called 'Q/R-site'. In GluR1, GluR3 and GluR4, a glutamine (Q) resides at this position. The gene for GluR2 also codes for a glutamine, but virtually all of the GluR2 mRNA is edited so that this glutamine is replaced with an arginine (R) [4]. In addition to Ca²⁺ permeability, the Q/R site influences the sensitivity of the receptor complex to blockage by polyamine spider toxins and internal polyamines [5,6].

Horizontal cells are interneurons of the retina contributing to the processing of light stimuli in the outer plexiform layer. These cells are tonically depolarized in the dark by L-amines [5,6]. Horizontal cells were enucleated and hemisected after the fish had been decapitated. The isolated retina was cut into 8–12 pieces and incubated for 20 min at room temperature in 4 ml Hank’s solution (in mM: 120.0 NaCl, 3.0 KCl, 2.0 CaCl₂, 1.0 MgSO₄, 1.0 Na-pyruvate, 1.0 NaH₂PO₄, 0.5 NaHCO₃, 8–12 pieces and incubated for 20 min at room temperature in 4 ml Hank’s solution (DMSO 0.5%). Joro spider toxin was directly dissolved in DMSO and diluted to their final concentrations in Ringer’s solution (DMSO <0.5%). Joro spider toxin (JSTX-3) and pentobarbital were directly dissolved in Ringer’s solution. The pH value for all solutions was adjusted to 7.4 with NaOH. All the drugs were purchased from Sigma (St Louis, Missouri, USA).

**MATERIALS AND METHODS**

**Isolation of horizontal cells:** Horizontal cells were enzymatically dissociated from the retinas of adult carp (Carassius auratus, 15–20 cm body length). The experimental procedure followed that described in a previous report [10]. Briefly, the eyes were enucleated and hemisected after the retina had been decapitated. The isolated retina was cut into 8–12 pieces and incubated for 20 min at room temperature in 4 ml Hank’s solution (in mM: 120.0 NaCl, 3.0 KCl, 0.5 CaCl₂, 12.0 Hepes, 1.0 MgSO₄, 1.0 Na-pyruvate, 1.0 NaH₂PO₄, 0.5 NaHCO₃, 20.0 Hepes and 16.0 glucose) containing 25 U/ml papain (E. Merck, Darmstadt, Germany) activated by 1 mg/ml L-cysteine (Bo’ao, Shanghai, China). After being rinsed, the retinal pieces were stored in Hank’s solution at 4°C until use. To obtain dissociated horizontal cells, the retina pieces were gently triturated with fire-polished glass pipettes in Ringer’s solution (in mM: 120.0 NaCl, 5.0 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 10.0 Hepes and 16.0 glucose). AMPA, nifedipine, ryanodine and thapsigargin were prepared in dimethyl sulfoxide (DMSO) and diluted to their final concentrations in Ringer’s solution (DMSO <0.5%). Joro spider toxin (JSTX-3) and pentobarbital were directly dissolved in Ringer’s solution. The pH value for all solutions was adjusted to 7.4 with NaOH. All the drugs were purchased from Sigma (St Louis, Missouri, USA).

**Measurement of intracellular Ca²⁺ concentration:** The intracellular concentration of free Ca²⁺ ([Ca²⁺]ᵢ) was
measured using a fura-2 imaging system. Fura-2/AM (Sigma) was dissolved in DMSO (5 mM stock solution) and added to prepared cell suspension with a final concentration of 5 μM. The cells were incubated at room temperature for 10 min to allow adherence and fura-2 loading. The cells were then continuously superfused with Ringer’s solution at a rate of 1 ml/min for 10 min prior to experiment. Horizontal cells, identified as cone dominant type [7], were examined. Excitation light with wavelengths of 340 and 380 nm for Ca2+ imaging was supplied by a high-speed scanning polychromatic light source (Hamamatsu, Shizuoka, Japan). The 340 and 380 nm wavelengths were applied alternately, and relevant fluorescence image pairs (F340 and F380) were acquired every 5 s. [Ca2+]i was presented by the ratio values (F340/F380) in this study. Control of monochromator and shutter, together with data acquisition, were executed with Aquacosmos 1.2 software. Normalized data were presented in the form of mean ± SD in text and mean ± SE in illustrations. A paired t-test was performed to original ratio data for statistical analysis.

RESULTS
Existence of Ca2+-permeable AMPA receptors: The application of 100 μM AMPA caused changes in [Ca2+]i, with an initial transient increase to a peak level and a following decrease to a steady level in horizontal cells. The initial peaky transient is believed to be attributed to Ca2+ release from ryanodine-sensitive intracellular Ca2+ stores [10]. The steady phase should simply reflect Ca2+ flux through the plasma membrane. Thus, in the whole text, the ratio value of the steady level in Ca2+ dynamics was measured to calculate Ca2+ influx across the plasma membrane. The application of AMPA can depolarize the horizontal cell membrane and this depolarization can in turn activate voltage-dependent Ca2+ channels that are of the high-voltage-activated, dihydropyridine-sensitive type [11–13]. The effect of nifedipine (100 μM), an antagonist of dihydropyridine-sensitive Ca2+ channels [12], on [Ca2+]i increase was therefore examined during AMPA application. Figure 1A gives an example, which shows that the application of 100 μM AMPA induced a transient increase of the ratio value (z = 0.34), followed by a return to a shoulder level (z = 0.20). However, when AMPA application was superimposed on the preapplication of nifedipine, the increase of [Ca2+]i was attenuated, and the peak value and the shoulder level of ratio increase (z) were measured at 0.23 and 0.10, respectively. The inhibitory effect of nifedipine on the ratio change (measured by the shoulder level) was apparent, which was 50% in this case. Statistical data showed that the inhibitory effect that nifedipine exerted on this ratio change was 50 ± 7% (mean ± SD, n = 7, see also Fig. 3b). The effect could be reversed after the nifedipine was washed out, as illustrated in Fig. 1a. These results suggest that apart from the voltage-dependent Ca2+ channels, horizontal cells should contain other Ca2+ influx components causing [Ca2+]i increase.

Following the previous findings that horizontal cells express Ca2+-permeable AMPARs, it might be reasonable to infer that the residual [Ca2+]i increase during the application of nifedipine resulted from Ca2+ influx via Ca2+-permeable AMPA receptors. To examine this speculation, JSTX-3 (2 μM), a specific antagonist of Ca2+-permeable AMPA receptors, was coapplied with nifedipine [5]. It has been reported that JSTX-3 blocks AMPARs by a use-dependent mechanism, which is that channels must be open for the toxin to access the channel pore [14,15]. As illustrated in Fig. 1b, the preapplication of nifedipine and JSTX-3 effectively inhibited the AMPA-induced [Ca2+]i increase.
changes and the inhibitory effect was more significant in the third exposure (c) to AMPA than that of the second one (b). The increase of [Ca\textsuperscript{2+}] was almost eliminated by coapplication of nifedipine and JSTX-3, with the relative change being 3±2% [ratio increase of the third AMPA application (c) vs. that of the first one (a), mean ± SD, n=8, see also Fig. 3b]. This result suggests that Ca\textsuperscript{2+}-permeable AMPA receptors are expressed on the horizontal cell.

Coexistence of Ca\textsuperscript{2+}-permeable and Ca\textsuperscript{2+}-impermeable AMPA receptors: In order to investigate whether Ca\textsuperscript{2+}-impermeable AMPA receptors are expressed on horizontal cells, JSTX-3 was applied alone. The reason is that if any Ca\textsuperscript{2+}-impermeable AMPA receptors exist, the application of AMPA in the presence of JSTX-3 would still induce the increase of [Ca\textsuperscript{2+}], because of the membrane potential depolarization and consequent Ca\textsuperscript{2+} influx through the activated voltage-gated Ca\textsuperscript{2+} channel. As illustrated in Fig. 2A, the increase of [Ca\textsuperscript{2+}], triggered by AMPA was slightly attenuated in the presence of JSTX-3 as compared with that in normal Ringer’s solution. The inhibition was gradually developed, with the relative change in the presence of JSTX-3 being 58±14% (n=6, d compared with a, see also Fig. 3b). This result suggests that the horizontal cells express Ca\textsuperscript{2+}-impermeable AMPA receptors.

Pentobarbital, up to a concentration of 100 μM, has been reported to be a selective antagonist of Ca\textsuperscript{2+}-permeable AMPA receptors [16–18]. Thus, for confirming the coexistence of Ca\textsuperscript{2+}-permeable and Ca\textsuperscript{2+}-impermeable AMPA receptors on horizontal cells, pentobarbital at a concentration of 100 μM was applied. As shown in Fig. 2B, the increase of [Ca\textsuperscript{2+}], was slightly inhibited when 100 μM AMPA was applied in the presence of 100 μM pentobarbital. The inhibitory effect was not time dependent. The relative change in the presence of pentobarbital was 70±8% (compared with a, n=10, see also Fig. 3b).

One may argue that, in addition to the Ca\textsuperscript{2+} influx across the plasma membrane, an increase of [Ca\textsuperscript{2+}] can also induce the release of Ca\textsuperscript{2+} from the internal stores of horizontal cells [10,19]. To further avoid any contribution from the Ca\textsuperscript{2+} store triggered by AMPA, experiments were also performed under blockage of ryanodine receptor with 20 μM ryanodine and inhibition of Ca\textsuperscript{2+} reuptake into internal stores with 2 μM thapsigargin. The result showed that the peak response of the AMPA-induced [Ca\textsuperscript{2+}], increase was blocked by internal stores inhibition, which was similar to our previous finding [10] (Fig. 3a). Expectedly, no significant difference in the steady level of AMPA-induced [Ca\textsuperscript{2+}], changes was observed, in the presence and absence of ryanodine and thapsigargin (99±3% in the presence of these chemicals, mean ± SD, p>0.05, paired t-test, n=10, Fig. 3b). Statistical results also showed that the superimposed application of nifedipine or JSTX-3 could both inhibit the increase of [Ca\textsuperscript{2+}], triggered by AMPA, with relative changes of 45±6% and 48±4%, respectively (compared with the control, n=10, Fig. 3b).

**DISCUSSION**

In the present study, we found the coexistence of Ca\textsuperscript{2+}-permeable and Ca\textsuperscript{2+}-impermeable AMPARs on carp retina horizontal cells. The application of Ca\textsuperscript{2+}-permeable AMPAR antagonist JSTX-3 and Ca\textsuperscript{2+}-impermeable AMPAR inhibitor pentobarbital could both attenuate the [Ca\textsuperscript{2+}], increase induced by exogenous application of AMPA, by directly or indirectly affecting the Ca\textsuperscript{2+} influx pathways. These effects persisted even when the intracellular Ca\textsuperscript{2+} stores were inhibited.

Ca\textsuperscript{2+}-permeable AMPARs are also expressed ubiquitously in many other neurons and glial cells. Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+}-permeable AMPARs served a crucial role in synaptic plasticity, such as synaptic strengthening in the dorsal horn neurons of the spinal cord [14], long-term potentiation in glutamatergic synapse in the amygdale [20] and long-term depression in CA3 hippocampal interneurons [21]. In retinas, Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+}-permeable AMPARs is also involved in some modulatory processes. It was previously found that Ca\textsuperscript{2+} influx via Ca\textsuperscript{2+}-permeable AMPARs was responsible for the retracting initiation of horizontal cell spinule, which was formed during light adaptation and retracted during dark adaptation in carp retinas [8].

Although the Ca\textsuperscript{2+} influx through Ca\textsuperscript{2+}-permeable AMPARs contributed to synaptic plasticity, it is also found that sustained Ca\textsuperscript{2+} influx through glutamate receptors is closely related to neuronal impairment and degeneration [22]. On the other hand, expression of Ca\textsuperscript{2+}-impermeable GluR2(R) channels blocked Ca\textsuperscript{2+} permeability of AMPARs and rescued vulnerable CA1 pyramidal neurons from delayed neurodegeneration following global ischemia, which demonstrate that Ca\textsuperscript{2+} entry through Ca\textsuperscript{2+}-permeable receptors.

**Fig. 3.** (a) Effect of ryanodine and thapsigargin in AMPA-triggered intracellular concentration of free Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]) dynamics. Preapplication of ryanodine and thapsigargin blocked the peak response, and the steady level of the AMPA-triggered [Ca\textsuperscript{2+}], changes was kept unaltered. The dotted lines indicate the steady level of AMPA-triggered [Ca\textsuperscript{2+}], increase. (b) Normalized data of the AMPA-induced [Ca\textsuperscript{2+}], increase, in the presence of various chemicals (mean ± SE), with and without the inhibition of intracellular Ca\textsuperscript{2+} stores by ryanodine and thapsigargin. *p<0.05 (paired t-test). Bars show the standard errors. Nif, nifedipine; Rya, ryanodine; TS, thapsigargin.
AMPARs determines neuronal sensitivity to ischemic insult [23]. Thus, Ca\(^{2+}\)-impermeable AMPARs seem to play a protective role in neurons. In the outer plexiform layer of the retina, horizontal cells are postsynaptic to photoreceptors that tonically release glutamate, which could induce some toxic effect in horizontal cells [24]. The expression of Ca\(^{2+}\)-impermeable AMPARs on horizontal cells may be responsible for reducing the effect of glutamate-induced toxicity, while maintaining the glutamatergic signal transmission. While the physiological significance of the coexistence of Ca\(^{2+}\)-permeable and Ca\(^{2+}\)-impermeable AMPARs on retinal horizontal cells still needs investigation, Liu and Cull-Candy [25] recently described a new form of synaptic plasticity – a rapid and lasting change in the subunit composition and Ca\(^{2+}\) permeability of AMPARs at cerebellar stellate cell synapses following synaptic activity. Their experiments show that the activity-induced Ca\(^{2+}\) influx through GluR2-lacking AMPARs controls the targeting of GluR2-containing AMPARs, implying the presence of a self-regulating mechanism [25]. Thus, the coexpression of Ca\(^{2+}\)-permeable and Ca\(^{2+}\)-impermeable AMPARs should endow the horizontal cells with the changeability in information transmission, and at the same time prevent the cell from glutamate-induced calcium toxicity and degeneration.

**CONCLUSION**

Two subtypes of AMPA receptors, Ca\(^{2+}\)-permeable and Ca\(^{2+}\)-impermeable, are coexpressed on dissociated carp retinal horizontal cells.

**REFERENCES**


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